

This Page Is Inserted by IFW Operations
and is not a part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

**As rescanning documents *will not* correct images,
please do not report the images to the
Image Problem Mailbox.**



Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number:

0 066 400

A1

(12) EUROPEAN PATENT APPLICATION

(21) Application number: 82302484.9

(51) Int. Cl.³: C 07 D 493/10

(22) Date of filing: 17.05.82

C 12 P 17/18, A 61 K 31/35
//(C07D493/10, 311/00, 303/00),
(C12P17/18, C12R1/645)

(30) Priority: 19.05.81 US 265135

(71) Applicant: WARNER-LAMBERT COMPANY

201 Tabor Road
Morris Plains New Jersey 07950(US)

(43) Date of publication of application:
08.12.82 Bulletin 82/49

(72) Inventor: Bloem, Russell Jack
4117 Woodworth
Dearborn Michigan 48126(US)

(84) Designated Contracting States:
AT BE CH DE FR GB IT LI LU NL SE

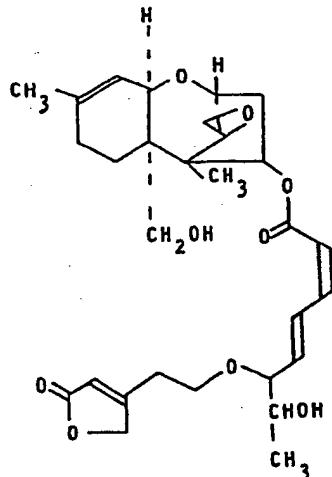
(72) Inventor: Bunge, Richard Harold
35180 Dumbarton
Mt. Clemens Michigan 48045(US)

(72) Inventor: French, James Clark
3150 Rumsey
Ann Arbor Michigan 48105(US)

(74) Representative: Jones, Michael Raymond et al.
Haseltine Lake & Co. 28 Southampton Buildings
Chancery Lane
London WC2A 1AT(GB)

(54) Antibiotic roridin L-2 and its production.

(57) There is disclosed a novel trichothecene antibiotic, roridin L-2, and a process for its production. The compound roridin L-2 has the following formula:



The compound roridin L-2, which may be formed by cultivating a suitable strain of *Myrothecium roridum*, NRRL 42202, has antimicrobial and antifungal activity.

EP 0 066 400 A1

-1-

"ANTIBIOTIC RORIDIN L-2 AND ITS PRODUCTION"

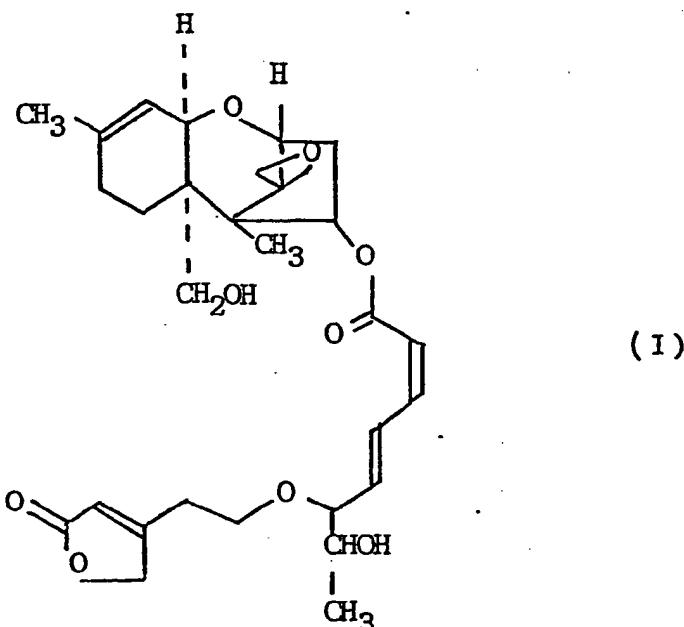
This invention relates to a novel trichothecene antibiotic compound, roridin L-2, which is represented by the following structural formula I, and to a process for the production of the compound:-

5

10

15

20



25

The process aspect of the present invention relates to a fermentation process for the production of the compound of the present invention by cultivating a roridin L-2 producing strain of an organism belonging to the species Myrothecium roridum, especially a strain closely resembling Myrothecium roridum Tode.

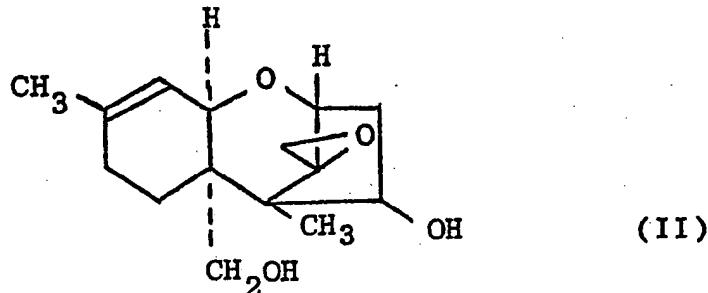
In addition, the present invention provides pharmaceutical compositions containing in addition to a pharmaceutically acceptable carrier, the compound

of the invention alone or in combination with another trichothecene derivative or other antitumor agents in the treatment of bacterial infections and neoplastic diseases. A review of the trichothecene antibiotics 5 is found in C. Tamm, "Progress in the Chemistry of Natural Products," 31, 64-117 (1974).

Mild alkaline hydrolysis of roridin L-2 produces verrucarol which has the following formula:-

10

15



a dihydroxysesterpene which is a structural unit 20 of several trichothecenes. Roridin L-2 can be readily distinguished from related, previously disclosed trichothecenes such as roridins A, D, E, E-2, H, J and K by the following characteristic properties exhibited by roridin L-2:

25 (1) an infrared absorption at 1780 cm^{-1}

(2) a proton magnetic resonance signal that appears, in deuterated chloroform solution, as a doublet ($J = 2\text{ Hz}$) centred at 4.80 ppm downfield from tetramethylsilane; and

30 (3) three ^{13}C magnetic resonance signals that appear, in deuterated chloroform solution, at 174.0 , 167.4 and 166.5 ppm downfield from tetramethylsilane.

The above spectral properties are not shown by any of 35 the known trichothecenes and clearly establish roridin L-2 as a new member of the trichothecene family of antitumor antibiotics.

-3-

In accordance with one aspect of the present invention, roridin L-2 is produced by cultivating a selected roridin L-2 producing strain of an organism that closely resembles Myrothecium roridum Tode under artificial conditions in a suitable nutrient medium until a substantial quantity of roridin L-2 is formed, which may then be isolated in pure form by procedures described hereinafter.

A new strain of a Myrothecium roridum species, suitable for the purpose of the present invention, has been isolated from a sample of soil collected in Cleveland, Ohio, United States of America. It corresponds morphologically to the description of this species by N.C. Preston, "Transactions Brit. Mycological Soc.," 26, 158 (1943); and J.C. Gilman, "Manual of Soil Fungi," Second Edition 1957, The Iowa State College Press, Ames, Iowa, United States of America. Cultures of this novel microbial strain have been deposited with the United States Department of Agriculture, Northern Utilization Research and Development Division, Peoria, Illinois, United States of America, and are being maintained in their permanent culture collection as NRRL 12303.

Cultural characteristics showed that the new Myrothecium roridum strain, NRRL 12303, differed slightly from the reference Myrothecium roridum Tode in the aerial and submerged mycelia.

The following table describes the mycelial characteristics of the two organisms in the different standard microbiological media: PDA (Potato Dextrose Agar), SDA (Sabouraud's Dextrose Agar), CZP (Czapek Dox Agar), and BHI (Brain Heart Infusion Agar).

-4-

Cultural Characteristics of 7-Day Cultures

Type of Mycelia	Media	Strain NRRL 12303	<i>Myrothecium roridum</i> Tode
5	PDA	Very variable: white, fluffy, slight yellow to dark green or black	Black with white edges
	Aerial Mycelia	White to orange yellow to dark green or black	White to slight pink-yellow, to dark green or black
	CZP	White to pink with black tufts	White to slight pink with black tufts
10	BHI	White to slight pink	White to slight pink
	PDA	Yellow orange to greyish green	Dark green with orange yellow edges
	SDA	Yellow orange with tufts of black	Yellow orange with tufts of black
	CZP	Yellow orange with tufts of black	Black with yellow orange edge
20	BHI	Orange yellow	Yellowish orange

25

30

35

The antitumor antibiotic compound, roridin L-2, is produced by the fungus during aerobic fermentation under controlled conditions. The fermentation medium consists of suitable sources of carbon, nitrogen, 5 inorganic salts, and growth factors assimilable by the microorganism. Examples of carbon sources are various sugars such as cerelose, lactose, and maltose; starch; dextrin; corn meal; and glycerol. The quantity of the carbon sources usually is in the range 10 from 0.5 to 6% by weight, but levels outside this range can also be used.

The sources of nitrogen can be organic, inorganic, or mixed organic-inorganic in nature. Examples of nitrogen sources that can be used in the culture 15 medium are cottonseed meal, corn germ flour, soybean meal, corn steep liquor, distillers' solubles, peanut meal, fish meal, peptonized milk, and various ammonium salts. The amount added usually is in the range from 0.1 to 3%, but higher amounts are also acceptable.

20 The inclusion of certain amounts of minerals and growth factors in the fermentation medium is also helpful in the production of roridin L-2. Crude medium ingredients such as distillers' solubles, corn steep liquor, fish meal, yeast products, peptonized 25 milk, and whey contain minerals and growth factors. However, inorganic salts such as potassium phosphate, sodium chloride, ferric sulphate, calcium carbonate, cobalt chloride, and zinc sulphate can be added to the fermentation medium.

30 The preferred process for producing roridin L-2 by using Myrothecium roridum is by submerged fermentation. According to this preferred process of the present invention, fermentation ingredients are prepared in solution and sterilized by autoclaving or steam heating. 35 The pH of the aqueous medium is preferably in the range from 6 to 8. The fermentation medium is cooled to a suitable temperature, generally in the range from

20 to 45°C, and then inoculated with the suitable culture. Fermentation is carried out with aeration and agitation, and the maximum production of roridin L-2 is usually reached in three to eight days.

5 In the submerged culture method, fermentation is carried out in shake flask or in stationary vat fermentors. In shake flasks, aeration is brought about by agitation of the flask which causes mixing of the medium with air. In the stationary fermentors, 10 agitation is provided by impellers in the form of a disc turbine, vaned disc, open turbine, or marine propeller; and aeration is accomplished by injecting air or oxygen into the fermentation mixture.

15 The examples which follow illustrate the preferred methods by which the product, roridin L-2, of the present invention may be obtained. The described process is capable of wide variation, and the present invention is not limited to the described process.

20 Fermentation in 200-Gallon (757 litre) Fermentors

A. Seed Development

25 A culture of the microorganism Myrothecium roridum NRRL 12303, preserved in a soil tube, was transferred to CIM 23 agar slants and incubated at 28°C for seven days.

CIM-23 Slant Medium

	Amidex corn starch	10 g
	N-Z Amine, type A	2 g
	Beef extract (Difco)	1 g
30	Yeast extract (Difco)	1 g
	Cobalt chloride · 6H ₂ O	20 mg
	Agar	20 g
	Distilled water	1,000 ml

35 The microbial growths in two slants were scraped, suspended in distilled water, and used to inoculate a 30 litre seed fermentor. The seed fermentor was prepared by charging it with 16 litres of ARM 1558A

-7-

medium and then autoclaving for 90 minutes at 121°C and 15 pounds per square inch gauge (103.4 kiloPascals).

ARM 1558A

	Cerelose	2.0%
5	Pharmamedia	0.3%
	Defatted corn germ flour	0.1%
	Soybean meal	0.1%
	CaCO ₃	1.0%
	K ₂ SO ₄ ·7H ₂ O	0.1%
10	MgSO ₄ ·7H ₂ O	0.1%
	NaCl	0.05%
	FeSO ₄ ·7H ₂ O	0.0001%

Use tap water, no pH adjustment.

B. Production Fermentation

15 Two 220 gallon (757 litre) fermentation tanks were used for producing roridin L-2. Each of these fermentors contained 160 gallons (606 litres) of presterilized ARM 1558 medium.

ARM 1558 Medium

20	Cerelose	5.0%
	Pharmamedia	0.3%
	Defatted corn germ flour	0.1%
	Soybean meal	0.1%
	CaCO ₃	1.0%
25	K ₂ HPO ₄	0.1%
	MgSO ₄ ·7H ₂ O	0.1%
	NaCl	0.05%
	FeSO ₄ ·7H ₂ O	0.0001%

Use tap water, no pH adjustment.

30 Each fermentor was inoculated with about 15 litres of the microbial growth from a 30 litre seed fermentor. The inoculated fermentors were stirred at 190 rpm at 30°C and sparged with 20 CFM (cubic feet per minute, equivalent to 566 litres per minute) of air for 119 hours. Dow Corning antifoam "C" was used to control foaming as required.

Production of roridin L-2 in the fermentation

broth was assayed vs KB and L1210 tissue cell lines. An aliquot of the fermentation broth was mixed with growing cultures of KB and L1210 cells to give a final dilution of the fermentation broth of 1:100. Activity was expressed as the percent growth of the cells relative to the control (no fermentation broth added). A fermentation broth that gave percent growth values of 0-25% for KB and 0-50% for L1210 was considered active.

The activities of the fermentation broths after 119 hours of fermentation are:

	<u>Inhibitory Zone (mm)</u>		<u>Cytotoxicity (% growth)</u>	
	<u>Saccharomyces</u>	<u>Candida</u>	<u>L1210</u>	<u>KB</u>
	<u>cerevisiae</u>	<u>albicans</u>		
15	16	15	4	0
	15	17	4	9

Isolation of Roridin L-2

The fermentation beers (a total of 1350 litres) from two 200 gallon (757 litre) fermentors prepared as described above were combined and mixed vigourously with 675 litres of ethyl acetate at pH 6.6 for one hour. Celite 545 (91 kg) was then added and the stirred mixture was filtered using a 75.8 cm plate and frame filter press. The filter cake was washed with 245 litres of ethyl acetate followed by 188 litres of deionized water. The filtrate and filter cake washes are combined and allowed to stand to permit the separation of phases. The upper organic phase amounted to about 660 litres; the lower aqueous phase (1520 litres) was separated and mixed with 340 litres of fresh ethyl acetate. After standing, the layers were separated and the upper organic layer (356 litres) was added to the first ethyl acetate extract. This combined ethyl acetate extract (1015 litres) was then concentrated in vacuo to 13.2 litres. The concentrated extract was dried using anhydrous sodium sulphate and then filtered. The solvent was removed in vacuo to leave approximately 900 g of an oily residue. This product,

hereinafter referred to as residue A, was processed by the fermentation method described below to afford a pure sample of roridin L-2.

Purification Method

5 A solution of 25g of residue A in 25 ml of methylene chloride was fractionated using a Prep LC/System 500 apparatus fitted with one Prep Pak 500 /silica gel cartridge (5.7 cm x 30 cm), both of which are available from Waters Instruments, Inc.,
10 Milford, Massachusetts, U.S.A. After injection of the charge, the silica gel column was eluted consecutively with 2000 ml of methylene chloride, 2500 ml of 75:25 methylene chloride:ethyl acetate, 3000 ml of 50:50 methylene chloride:ethyl acetate, 1000 ml of 25:75
15 methylene chloride:ethyl acetate and finally with 1000 ml of ethyl acetate. Analysis of each fraction by means of high performance liquid chromatography (HPLC) using a 0.39 cm (I.D.) x 30 cm μ Bondpak (C₁₈ silica gel) column, a solvent system consisting of 60:40 methanol:water, and UV detection at 254 nm showed that most of the fractions eluted with 50:50 methylene chloride:ethyl acetate contained the desired material, roridin L-2. The retention time of this compound in the above analytical HPLC system was
20 approximately 3.2 minutes using a flow rate of 2 ml/ minute. The fractions (2000 ml) containing roridin L-2 were combined and concentrated in vacuo to leave an oily residue, residue B (4.2 g).

Residue B was combined with 1.8 g of equivalent
30 material obtained from a previous Prep LC fractionation and dissolved in 21 ml of 70:30 methanol:water. One-third portions of this solution were then subjected to further fractionation by low pressure chromatography over 250 g of C₁₈ silica gel (Waters Instrument, Inc.), packed in a 4.8 cm (O.D.) x 45 cm Michel-Miller column (available from Ace Glass Co., Vineland, New Jersey, U.S.A.) using an eluant consisting of 50:50

-10-

methanol:water. Between chromatographic runs, the C₁₈ silica gel column was washed with methanol and reequilibrated with 50:50 methanol:water before the next charge was introduced. Fractions were monitored using the analytical HPLC conditions described above. The 5 fractions that contained only roridin L-2 were combined and concentrated in vacuo to remove methanol. The remaining aqueous phase was extracted with two 500 ml portions of ethyl acetate. The organic extracts were 10 combined, washed with 200 ml of water, dried over anhydrous sodium sulphate, and then filtered. Removal of the ethyl acetate by concentration of the filtrate in vacuo afforded 3.4 g of pure roridin L-2 as a white solid. The properties which defined roridin L-2 as a novel 15 compound are listed below.

Properties of Roridin L-2

Ultraviolet Absorption Spectrum in Methanol, as represented in Figure 1 of the accompanying drawings:-

	<u>λ_{max}</u>	<u>E_{1cm}</u>
20	259 nm	465

Infrared Absorption Spectrum in KBr, as represented in Figure 2 of the accompanying drawings:-

25 Principal absorptions at: 3450, 2970, 1782, 1750, 1640, 1600, 1435, 1284, 1180, 1084, 1030 and 965 reciprocal centimetres.

Optical Rotation:-

$$[\alpha]_D = 83.6^\circ \text{ (1.0% in chloroform)}$$

Elemental Analysis:-

30 1. dried 18 hours at 25°C/ 1.33 Pascals

	<u>% C</u>	<u>% H</u>	<u>% O</u>
Calcd. for C ₂₉ H ₃₈ O ₉ .0.5 H ₂ O	64.55	7.29	28.17
Found:	64.68	7.12	28.70
	64.41	7.15	28.95

35 2. dried for 2 hours at 100°C/ 13.3 Pa and then for 4 hours at 50°C/ 13.3 Pa

		<u>% C</u>	<u>% H</u>
	Calcd. for C ₂₉ H ₃₈ O ₉ :	65.64	7.22
	Found:	65.21	6.93
		65.36	7.28

5 Proton Magnetic Resonance Spectrum, 2% in CDCl₃, as represented in Figure 3 of the accompanying drawings:

Principal signals at:

(s = singlet, d = doublet, t = triplet,
m = multiple) 0.83s, 1.16d, (J = 6), 1.72s, 2.02m,
10 2.81d (J = 4), 3.13d (J = 4), 3.5-4.0 (complex signals),
4.80d (J = 2), 5.49 broad d (J = 6), 5.22-6.02
(complex signals), 6.08 dd (J = 4,6), 6.60t (J = 11),
and 7.60 dd (J = 11,15) parts per million downfield
from tetramethylsilane.

15 Carbon-13 Nuclear Magnetic Resonance Spectrum in CDCl₃, as represented in Figure 4 of the accompanying drawings:-

The principal signals, in parts per million downfield from TMS, are at:

20	Signal	Ppm (downfield from	Signal	Ppm (downfield from
	Number	TMS)	Number	TMS)
	1	173.95	18	73.46
	2	167.43	19	69.74
	3	166.46	20	66.83
25	4	143.37	21	66.34
	5	140.57	22	65.59
	6	138.89	23	62.51
	7	130.53	24	48.86
	8	118.88	25	48.00
30	9	118.72	26	44.28
	10	116.67	27	36.19
	12	85.38	28	29.23
	13	78.91	29	28.04
	14	78.42)	30	23.19
35	15	77.02) CDCl ₃	31	21.14
	16	75.62)	32	18.44
	17	75.40	33	6.57

-12-

High Performance Liquid Chromatography

Column: μ Bondpak C₁₈ (3.9 mm I.D. X 30 cm)
 System: 60:40 methanol:water
 Flowrate: 2 ml/min
 5 Detection: Ultraviolet absorption at 254 nm
 Retention time: 3.2 min

Antitumor Activity of Roridin L-2 Against P388

Lymphatic Leukemia in Mice

10	Dose (mg/kg/day)	T/C* percent	
		MST test 1	MST test 2
	16	---	185
	8	157	176
	4	134	132
15	2	140	144
	1	132	150

*T/C percent MST = 100 x median survival time in days of treated/control mice.

20 Values 130 were considered active. The test method used was based on that described in Cancer Chemother. Reports 3: 1-87 (part 3), 1972.

25 The ID₅₀ of Roridin L-2 against L1210 cells was 0.178 μ g/ml. The zone diameter of inhibition of Roridin L-2 against KB cells in agar was 28 mm when a 6.4 mm paper disc moistened with a solution containing 100 μ g of Roridin L-2 per millilitre was used; and 12 mm when a 6.4 mm paper disc moistened with a solution containing 10 μ g of Roridin L-2 per millilitre was used.

30 The antibiotic roridin L-2 can be used for its antimicrobial and antitumor activity in the form of pharmaceutical compositions containing roridin L-2 and a compatible pharmaceutically acceptable carrier. The compositions may also contain other active antibacterial and/or antitumor agents. The

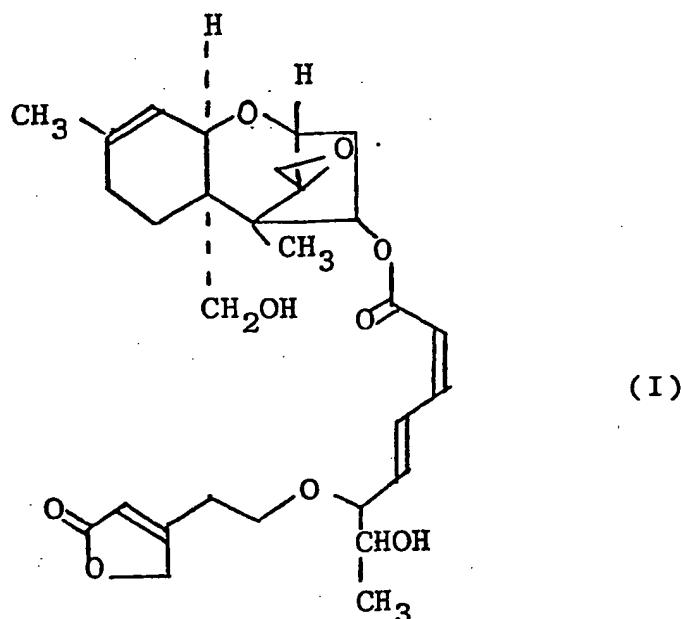
-13-

compositions may be made up in any pharmaceutical form appropriate for the route of administration intended. Examples of such compositions include solid compositions for oral administration such as tablets, capsules, 5 pills, powders and granules, liquid compositions for topical or oral administration such as solutions, suspensions, syrups and elixirs, and preparations for parenteral administration such as sterile solutions, suspensions, or emulsions.

10 For use as an antibacterial agent, the compositions are administered so that the concentration of active ingredient is greater than the minimum inhibitory concentration for the particular organism being treated. A suggested dosage regimen for use as an antitumor 15 agent in mammalian species is 0.1 to 10 mg/m² for a single daily intravenous treatment course with roridin L-2. (The surface area of an adult male is approximately 1.7 square metres).

CLAIMS (for all States except Austria):

1. The antibiotic compound roridin L-2 having the following structural formula:-



2. A process for the production of the compound roridin L-2 as claimed in Claim 1, which process comprises cultivating a roridin L-2 producing strain of the organism Myrothecium roridum in a suitable medium until a substantial quantity of roridin L-2 is formed.

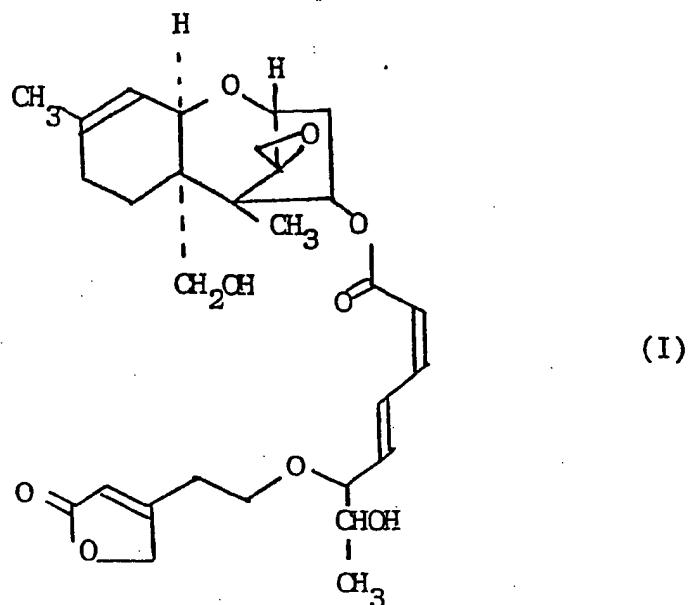
3. A process according to Claim 2, which further includes the step of isolating the compound roridin L-2 in pure form.

4. A process according to Claim 2 or 3, wherein the strain of Myrothecium roridum is that deposited as NRRL 12303.

5. A pharmaceutical composition comprising the antibiotic compound roridin L-2 as claimed in Claim 1, and a pharmaceutically acceptable carrier.

CLAIMS (for Austria only):

1. A process for producing the antibiotic compound roridin L-2 which has the following structural formula:



which process comprises cultivating a roridin L-2 producing strain of the organism Myrothecium roridum in a suitable medium until a substantial quantity of roridin L-2 is formed.

2. A process according to Claim 1, which further includes the step of isolating the compound roridin L-2 in pure form.

3. A process according to Claim 1 or 2, wherein the strain of *Myrothecium roridum* is NRRL 12303.

4. A process for producing a pharmaceutical composition, which comprises incorporating into a pharmaceutically acceptable carrier the compound roridin L-2 of formula I shown in Claim 1.

0066400

1/4

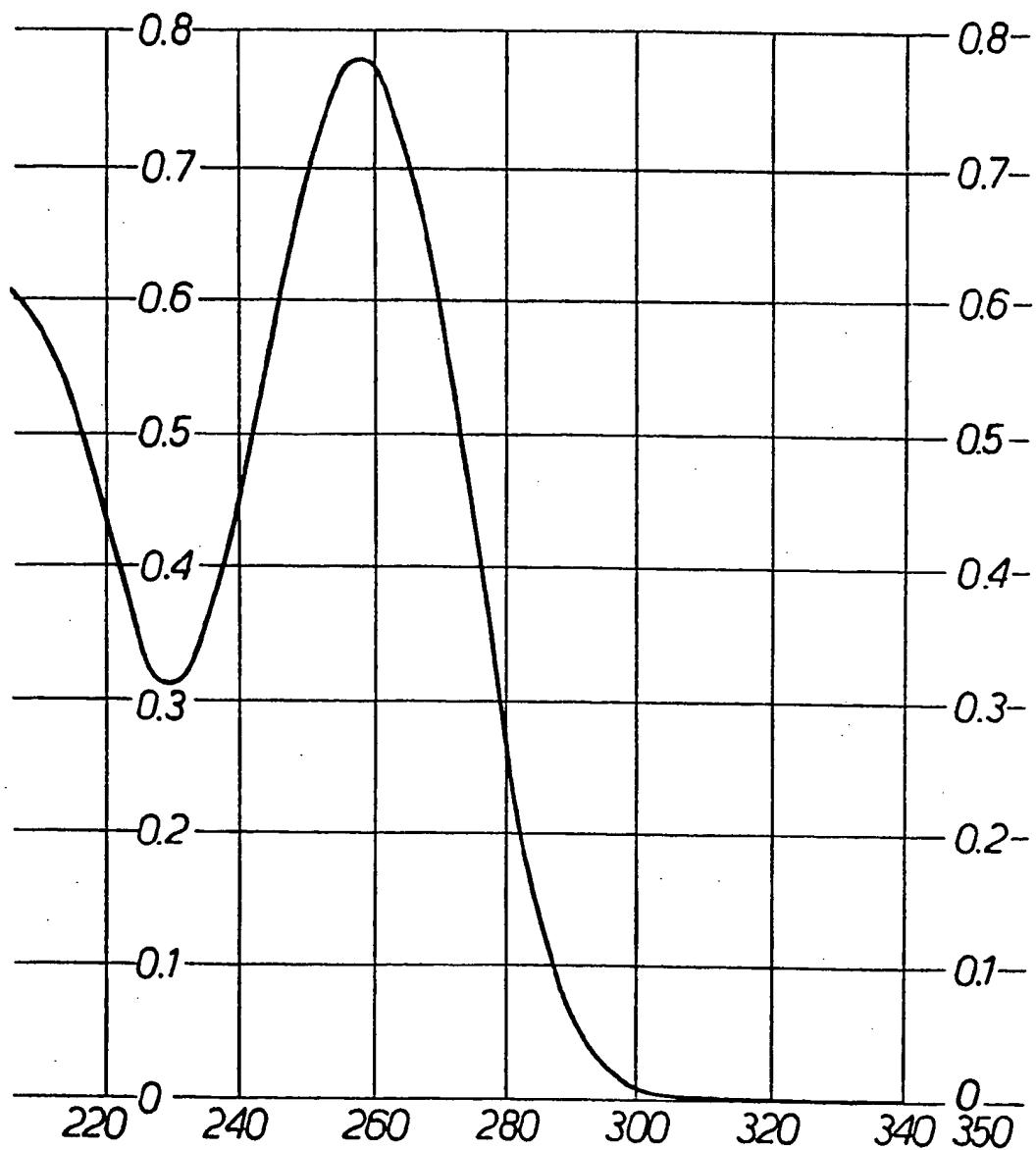


FIG.1.

0066400

2/4

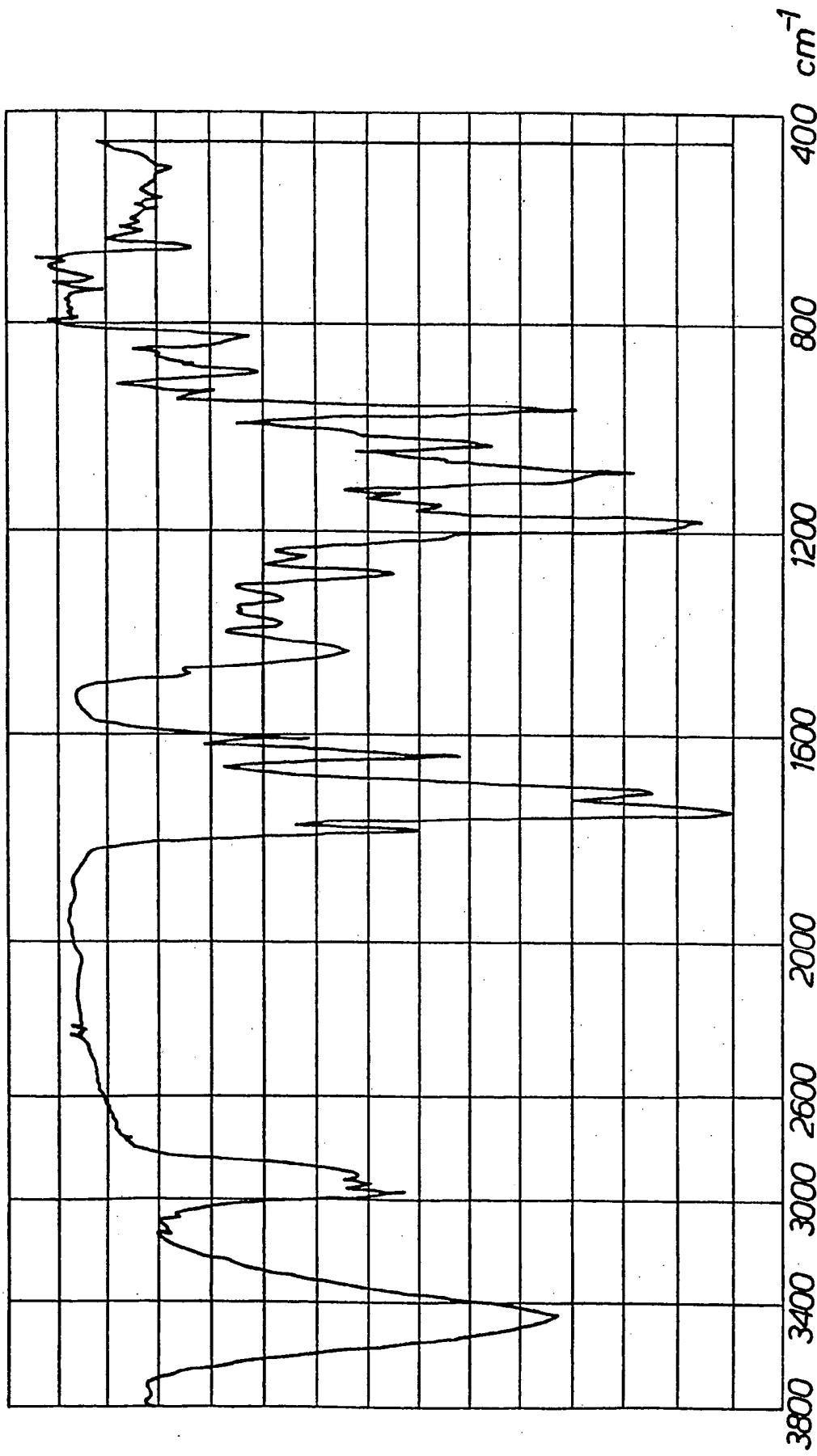


FIG. 2.

0066400

3/4

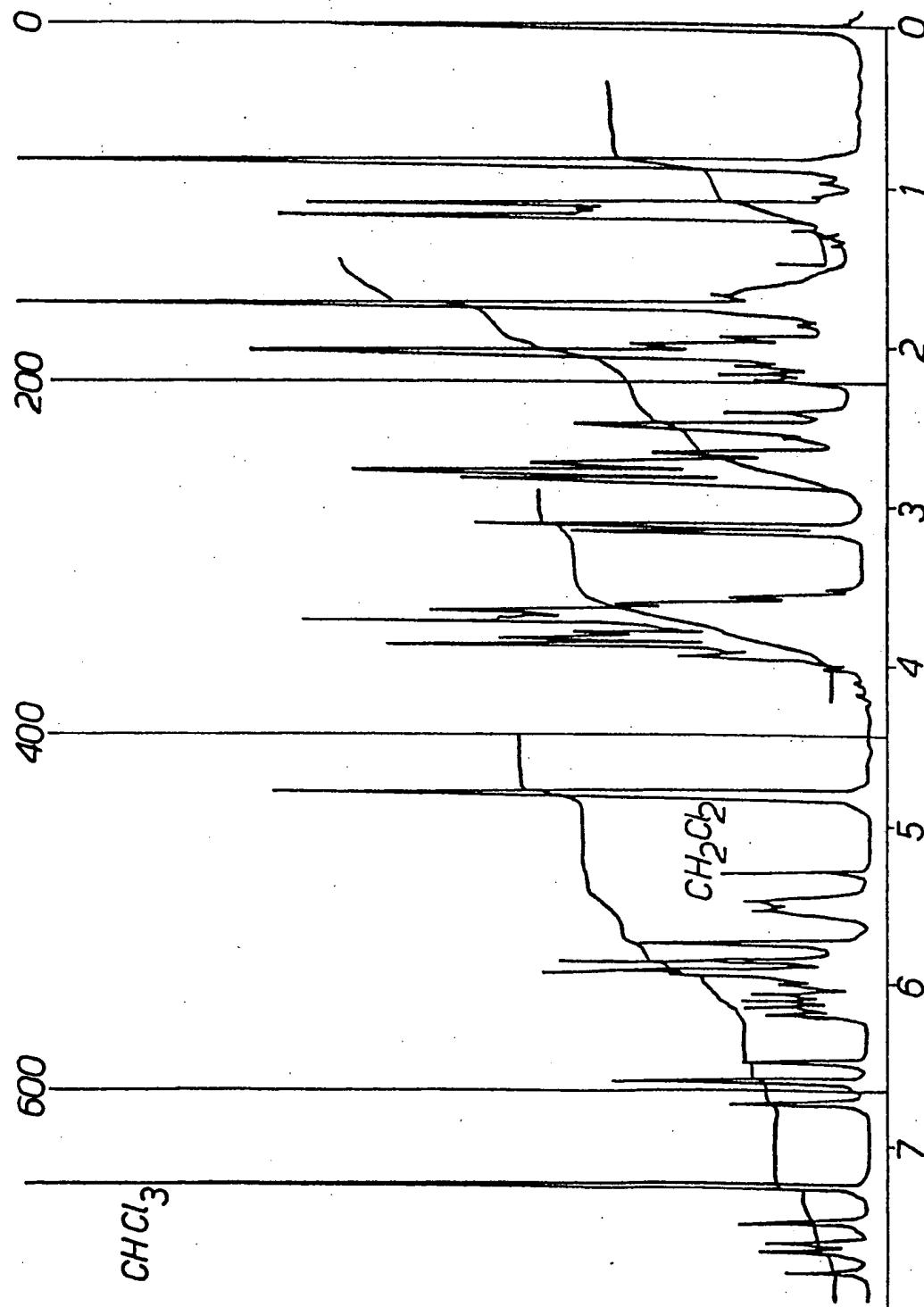


FIG. 3.

4/4

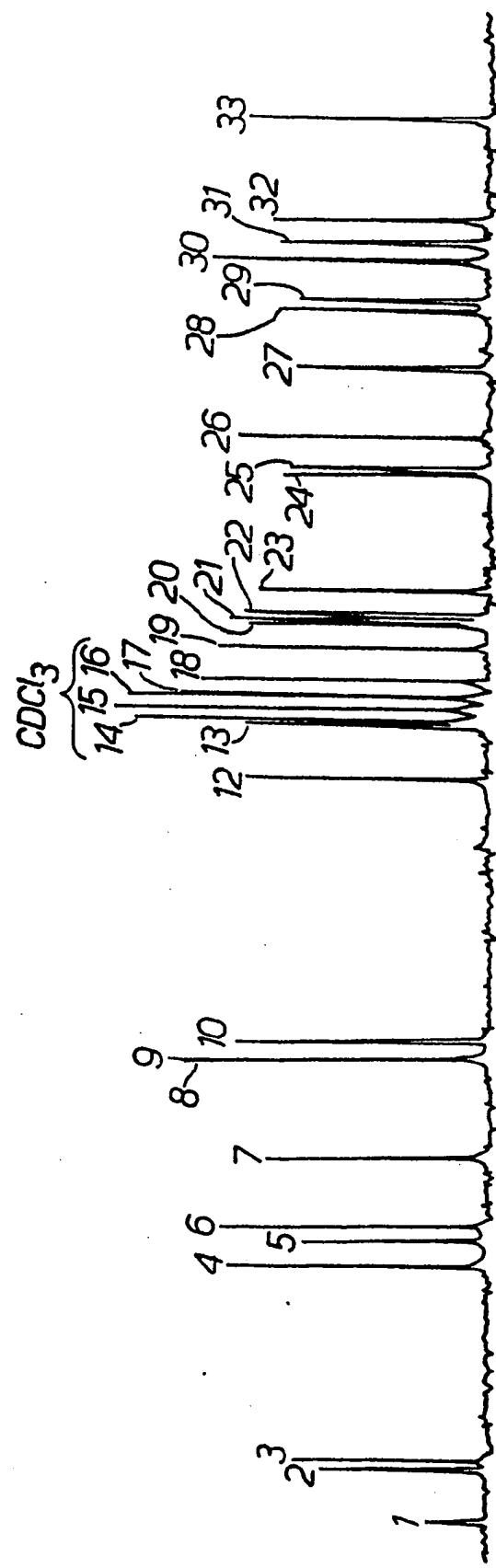


FIG. 4.



DOCUMENTS CONSIDERED TO BE RELEVANT					
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. *)		
P, X	THE JOURNAL OF ORGANIC CHEMISTRY, vol. 47, no. 6, 12th March 1982, pages 1117-1123; B.B.JARVIS et al.: "Isolation and characterization of the trichoverroids and new Roridins and verrucarins". *Page 1117,1118, "Chart I"; page 1119, "Chart II"; page 1121, line 12 from below and formula "i"*	1-5	C 07 D 493/10 C 12 P 17/18 A 61 K 31/35 (C 07 D 493/10 C 07 D 311/00 C 07 D 303/00) (C 12 P 17/18 C 12 R 1/645) //		
A	FR-A-1 348 003 (SANDOZ) *The whole document*	1,2,5			
A	US-A-4 244 874 (KANEKO) *The whole document*	1			
TECHNICAL FIELDS SEARCHED (Int. Cl. *)					
			A 61 K 31/00 C 07 D 493/00 C 12 P 17/00 C 12 R 1/00		
The present search report has been drawn up for all claims					
Place of search	Date of completion of the search	RAJIC M. Examiner			
THE HAGUE	12-08-1982				
CATEGORY OF CITED DOCUMENTS					
X : particularly relevant if taken alone	T : theory or principle underlying the invention				
Y : particularly relevant if combined with another document of the same category	E : earlier patent document, but published on, or after the filing date				
A : technological background	D : document cited in the application				
O : non-written disclosure	L : document cited for other reasons				
P : intermediate document	& : member of the same patent family, corresponding document				